Inhibition of growth of treponemes by antimicrobial agents

I. J. ABRAMSON AND R. M. SMIBERT*

Anaerobe Laboratory, Virginia Polytechnic Institute, and State University, Blacksburg, Virginia 24060, U.S.A.

The antimicrobial sensitivity of treponemes has been little investigated in comparison with that of micro-organisms that can be more easily cultured. Results of antibiotic sensitivity tests *in vitro* have been reported for oral treponemes in which inhibitory concentrations were determined by observing the growth of cultures (Fitzgerald and Hampp, 1952; Berger, 1956, 1958a, b, c, 1959, 1960; Hampp and Fitzgerald, 1959; Berger and Marggraf, 1960). Eagle and Musselman (1944) reported that a penicillin concentration of 0·1 to 0·25 units/ml. rendered 90 to 99 per cent. of Reiter treponemes non-viable in 8 to 12 hrs.

Inhibition of motility has been used to determine the sensitivity of living suspensions of *Treponema pallidum* and other pathogenic treponemes to antibiotics (Dunham, Hamre, McKee, and Rake, 1944; Nelson, 1948; Nell, 1954). The sensitivity of the non-pathogenic cultivable Nichols, Kazan, and Reiter treponemes to erythromycin was determined by observing the inhibition of motility of cells under darkfield microscopy (Keller and Morton, 1953).

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Penicillin has been the accepted treatment for syphilis since the early 1940s (Mahoney, Arnold, and Harris, 1943; Willcox, 1954; Yobs, Clark, Mothershed, Bullard, and Artley, 1968), but virulent *T. pallida* have recently been recovered from man and animals treated with presumably adequate concentrations of penicillin (Collart, Borel, and Durel, 1962; Yobs and others, 1968).

Until such time as the pathogenic species have been cultured in vitro, cultivable treponemes must serve as a model for determining the effect of antibiotics. The purpose of the present investigation was to determine the concentrations of antibiotics and other antimicrobial agents that inhibited growth of representative strains of various species of cultivable treponemes.

Material and methods

TREPONEME STRAINS

The strains used in these studies are listed in Table I.

†Bellco Glass, Inc., P.O. Box B, Vineland, New Jersey 08360, U.S.A.

FPfizer Diagnostics Division, New York, New York 10036, U.S.A. SDifco Laboratories, Detroit, Michigan 48201, U.S.A. Illipiacetyl tartaric acid ester of tallow monoglycerides, Witco Chemical Co. 277 Park Avenue, New York, New York 10017, U.S.A.

TABLE I Treponemes studied

Old name	New name	Origin	Source	Special requirements
Reiter Kazan T. refringens T. calligyrum Nichols (avirulent)	T. phagedenis T. phagedenis T. refringens T. refringens T. refringens	Genital-anal	CDC ^b CDC P° P CDC	Rabbit serum
T. microdentium (FM) T. ambiguum T-32-A T. vincentii (N-9)	T. denticola T. denticola T. denticola T. vincentii	Human oral cavity	NIH ^d P Dr. T. Rosebury NIH	Rabbit serum cocarboxylase
Human oral (HO) 5 HO 18 HO 27	a 	Human oral cavity	VPI ^e	Rumen fluid
Pig faeces (PF) 23 PF 31 PF 39 PF 28 PF 44		Pig faeces	VPI	Rumen fluid

Stock cultures were incubated 24 to 48 hrs. before inoculation into test media. Cell counts after incubation were 10^7 to 10^8 treponemes/ml., using a Petroff-Hausser Counting Chamber. Viable cell counts, using 10-fold dilutions of cultures, agreed with the microscopic cell counts. A stock culture was inoculated into 7.5 ml. pre-reduced test medium, giving a final cell count of 10^6 to 10^6 treponemes/ml. All cultures were incubated at 37° C. except *T. vincentii* which was incubated at 34° C.

MEDIA

Pre-reduced, anaerobic media with an oxidation-reduction potential of less than -150 mv. were prepared as described in the 'Outline of Clinical Methods in Anaerobic Bacteriology' (Anaerobe Laboratory, 1970). All inoculations were carried out under O₂-free nitrogen using the V.P.I. Anaerobic Culture System.†

Treponemes requiring serum were cultured in PYGS medium which contained peptone M‡, 2 g.; yeast extract§ 1 g.; dextrose 1 g.; agar 0·2 g.; ammonium sulphate 0·05 g.; soluble starch§ 0·05 g.; TEM-4T|| 0·016 g.; L-cysteine HCl hydrate 0·16 g.; sodium bicarbonate 0·5 g.; resazurin solution (25 mg. per cent.) 0·4 ml.; salt solution 50 ml. (anhydrous MgSO₄ 0·02 g.; CaCl₂·2H₂O 0·02 g.; K₂HPO₄ 0·1 g.; KHPO₄ 0·1 g.; NaCl 0·2 g. dissolved in 100 ml. distilled water); and distilled water 50 ml. The pH was adjusted to 6·5 before autoclaving and was 6·8 to 7·4 after sterilization. Sterile rabbit serum and cocarboxylase were added aseptically to autoclaved medium to final concentrations of 12 and 0·00012 per cent. respectively.

RABBIT SERUM

This was sterilized by filtration through a 0.45 μm . pore diameter membrane filter, aseptically dispensed into sterile glass bottles, heat inactivated at 58 to 60°C. for 4 hrs., and stored at -20°C.

COCARBOXYLASE

50 mg. were dissolved in 100 ml. distilled water and filtered through a 0·22 μ m. pore diameter membrane filter; 1 ml. of the sterile solution was added to 50 ml. sterile inactivated rabbit serum.

Treponemes requiring rumen fluid were cultured in human oral (HO) medium which consisted of peptone M 0.1 g.; yeast extract 0.1 g.; dextrose 0.5 g.; agar 0.2 g.; ammonium sulphate 0.05 g.; soluble starch 0.05 g.; Lcysteine-HCl 0·16 g.; sodium bicarbonate 0·5 per cent.; resazurin solution 0.4 ml.; salt solution 50 ml.; rumen fluid 28 ml.; and distilled water 22 ml. The pH was adjusted to 6.5 before autoclaving and was 6.8 to 7.4 after autoclaving. These cultures were maintained in E-medium, which consisted of peptone M 0.05 g.; yeast extract 0.05 g.; dextrose 0.14 g.; agar 0.2 g.; ammonium sulphate 0.05 g.; soluble starch 0.05 g.; L-cysteine-HCl 0.16 g.; sodium bicarbonate 0.5 g.; resazurin solution 0.4 ml.; salt solution 50 ml.; rumen fluid 28 ml.; and distilled water 22 ml. The pH was adjusted to 6.5 before sterilizing and was 6.8 to 7.4 after sterilizing.

RUMEN FLUID

Contents from the rumen of cattle were collected and filtered through two layers of cheesecloth. The liquid portion was siphoned into bottles, autoclaved at 121°C. at 15 lb. pressure for 15 min. and stored at 4°C. In preparing media, the rumen fluid was centrifuged at 10,000 G. for 20 min., added to the media, and sterilized by autoclaving.

All treponemes were grown in pre-reduced E-medium or in pre-reduced E-medium with 12 per cent. serum and 0.00012 per cent. cocarboxylase for 2 to 3 days and preserved at -85°C.

ANTIMICROBIAL AGENTS

All antibiotics and chemicals were diluted so that the final concentrations in 7.5 ml. culture medium were 0.01, 0.1, 1, 10, 100, 500, and 1,000 μg . or units/ml.

TABLE II Growth inhibition of treponemes by penicillins

Treponeme strains	Inhibitory concentrations of antimicrobial agents									
	Pend (units/ml.)	Ampe (µg./ml.)	Naf¹ (µg./ml.)	Οχ ^g (μg./ml.)	Cloxh (µg./ml.)	KPMP ¹ (µg./ml.)	Ceph ^j (µg./ml.)			
Reiter ^a	1	1	1	1	1	1	10			
Kazana	0.1	1	1	1	1	0.1	10			
T. refringens	0.1	1	10	10	1	0.1	0.01			
Calligyrumb	0.1	0.1	0.1	0.1	0.1	0.1	0.1			
Nichols ^b	1	0.1	1	0.1	0.1	1	0.1			
Microdentium FMc	0.1	0.1	1	0.1	0.1	0.1	0.1			
Ambiguum ^c	0.1	1	0.1	0.1	0.1	0.1	1			
Г-32-Ас	0.1	0.1	0.1	0.1	0.1	0.1	0.1			
T. vincentii N-9	0.1	1	1	1	0.1	0.1	0.1			
HO 5	10	100	100	100	100	500	0.1			
HO 18	100	10	100	100	100	500	1			
HO 27	100	100	500	500	100	500	1			
21 23	10	0.1	10	10	10	10	0.1			
₹¥ 31	10	100	10	1	10	1	1			
PF 39	10	10	10	10	10	1	1			
F 28	10	10	10	10	10	10	0.1			
PF 44	1	10	1	1	1	10	10			

T'. phagedenis, bT. refringens, cT. denticola, K penicillin, capacillin, nafcillin, coxacillin, coxacillin, K phenoxymethyl penicillin, cephalothin.

BACTERIOSTASIS

The bacteriostatic concentration of an antimicrobial agent was considered to be the lowest concentration in the dilution series in which no growth was observed after 3 days. All cultures were examined for visible growth and were also examined with a darkfield microscope (×1,000) on the 3rd day of incubation. Bacteriostatic activity was assessed by comparing the growth, cell morphology, and motility of treponemes in media containing the antimicrobial agent with those of treponemes in control media.

Results

Tables II to VI show the concentrations of antimicrobial agents that inhibited the growth of the treponemes studied. They were all apparently sensitive to cephalothin and most strains were apparently sensitive to the penicillin group of antibiotics except the human oral (HO) isolates (Table II).

Table III shows that all strains were apparently sensitive to bacitracin and erythromycin while most strains were sensitive to tylosin, lincomycin, and vancomycin. Treponeme strains varied in their sensitivity to novobiocin.

In Table IV, the tetracycline group of antibiotics were inhibitory to all treponemes in low concentrations except the strains isolated from pig faeces (PF-group). Treponemes were resistant to chloramphenicol.

Growth inhibitory concentrations of other antimicrobial agents to individual strains of treponemes were varied and are listed in Tables V and VI.

TABLE III Growth inhibition of treponemes by macrolides and other antibiotics

	Inhibitory con	centrations of antin	nicrobial agents				
Treponeme strains	Novod (µg./ml.)	vanco ^e (μg./ml.)	Bac ^t (μg./ml.)	Ery ^g (μg./ml.)	Tyloh (µg./ml.)	Linco¹ (µg./ml.)	
Reitera	100	1	1	0.1	100	100	
Kazan ^a	10	1	0.1	0.1	10	10	
T. refringens	100	1	0.01	0.01	10	1	
Calligyrum ^b	500	1	0.1	0.1	10	1	
Nichols ^b	500	0.1	0.1	0.01	1	1	
Microdentium FM ^c	100	1	1	0.01	100	10	
ambiguum ^c	10	1	1	0.1	10	1	
Г-32-А ^с	10	10	0.1	0.01	10	10	
^r . vincentii N-9	1	1	0.1	0.1	1	10	
HO 5	100	1	1	0.01	1	10	
HO 18	500	10	1	0.01	1	10	
HO 27	100	10	1	0.01	1	10	
PF 23	0.1	100	10	0.1	10	10	
PF 31	10	100	10	0.1	10	10	
F 39	1	500	10	0.1	10	10	
F 28	1	100	1	0.1	10	10	
PF 44	10	100	1	0.1	10	10	

aT. phagedenis, bT. refringens, cT. denticola, dnovobiocin, evancomycin, bacitracin, gerythromycin, btylosin, llincomycin.

TABLE IV Growth inhibition of treponemes by the tetracyclines and chloramphenicol

	Inhibitory concentrations of antimicrobial agents									
Treponeme strains	Tet ^d (µg./ml.)	Chlortete (µg./ml.)	Oxytet ^t (µg./ml.)	Dmethchg (µg./ml.)	Doxy ^h (µg./ml.)	Methac¹ (μg./ml.)	Chloram ^j (µg./ml.)			
Reitera	1	1	10	1	1	1	500			
Kazana	1	10	10	1	0.1	1	500			
T. refringens	1	10	1	1	0.1	0.1	100			
Calligyrum ^b	1	10	1	1	1	0.1	100			
Nichols ^b	1	10	1	1	1	1	100			
Microdentium FMc	1	10	1	1	1	1	100			
Ambiguum ^c	1	10	10	0.1	0.1	0.1	100			
T-32-A ^c	1	1	1	0.1	0.1	0.1	100			
T. vincentii N-9	1	1	1	0.1	0.1	0.1	100			
HO 5	10	10	10	10	10	1	100			
HO 18	1	10	1	1	1	1	100			
HO 27	1	10	1	1	1	1	100			
PF 23	100	500	500	100	100	100	100			
PF 31	100	100	100	100	100	100	100			
PF 39	100	500	100	100	100	100	100			
PF 28	100	100	100	100	500	500	10			
PF 44	500	500	500	100	500	100	100			

^aT. phagedenis, ^bT. refringens, ^cT. denticola, ^dtetracycline, ^echlortetracycline, ^foxytetracycline, ^gdemethylchlortetracycline, ^hdoxycycline, ^fmethacycline, ichloramphenicol.

TABLE	v	Growth inhibition	of tr	ebonemes	bν	aminoglycoside	and	peptide antibiotics

-	Inhibitory concentrations of antimicrobial agents									
Treponeme strains	Strep ^d (µg./ml.)	DHSMe (μg./ml.)	Kan ^t (μg./ml.)	Gent ^g (µg./ml.)	Neo ^h (µg./ml.)	Viom¹ (μg./ml.)	Tyro ^j (µg./ml.)			
Reitera	100	500	1,000	500	500	> 1,000	500			
Kazan ^a	500	100	1,000	ND	500	> 1,000	100			
T. refringens	10	1	100	ND	10	100	100			
Calligyrum ^b	10	1	100	ND	10	100	10			
Nicholsb	10	1	1	1	1	10	10			
Microdentium FM ^c	10	1	100	10	10	10	100			
Ambiguum ^c	100	10	100	ND	100	100	500			
T-32-Ac	100	100	100	ND	10	100	100			
T. vincentii N-9	10	100	100	ND	100	1,000	500			
HO 5	500	100	100	ND	500	> 1,000	10			
HO 18	500	100	500	ND	100	100	500			
HO 27	500	100	100	ND	100	100	10			
PF 23	1,000	1,000	100	ND	100	500	100			
PF 31	> 1,000	> 1,000	500	ND	100	1,000	500			
PF 39	1,000	1,000	100	ND	500	500	500			
PF 28	100	100	10	ND	100	500	500			
PF 44	100	100	100	ND	500	1,000	500			

aT. phagedenis, bT. refringens, cT. denticola, dstreptomycin, edihydrostreptomycin, skanamycin, sgentamicin sulphate, hneomycin, sviomycin tyrothricin, ND = not done.

TABLE VI Growth inhibition of treponemes by miscellaneous antibiotics and chemicals

Treponeme strains	Inhibitory concentrations of antimicrobial agents									
	Furaz ^d (µg./ml.)	Usnice (µg./ml.)	5-FU ^t (μg./ml.)	Tell ^g (µg./ml.)	Thall ^h (µg./ml.)	BG^i $(\mu g./ml.)$	CV ¹ (μg./ml.)			
Reiter ^a	100	1,000	500	100	100	500	100			
Kazan ^a	100	1,000	1,000	100	500	500	500			
T. refringens	100	100	> 1,000	100	100	100	100			
Calligyrumb	100	100	> 1,000	100	100	100	500			
Nichols ^b	100	100	> 1,000	100	500	100	100			
Microdentium FMc	100	100	100	100	500	100	500			
Ambiguum ^c	100	100	> 1,000	100	500	100	500			
T-32-A ^c	100	100	100	10	100	100	500			
T. vincentii N-9	10	500	100	10	100	100	100			
HO 5	10	10	10	500	10	100	500			
HO 18	100	100	100	100	100	10	100			
HO 27	100	100	100	100	100	100	100			
PF 23	500	10	> 1,000	100	100	100	100			
PF 31	500	100	> 1,000	500	100	100	100			
PF 39	500	10	> 1,000	100	100	500	100			
FP 28	> 1,000	10	> 1,000	100	100	10	10			
PF 44	500	100	> 1,000	100	100	500	100			

aTreponema phagedenis, bT. refringens, cT. denticola, durazolidone, cusnic acid, 15-fluorouracil, aK tellurite, hThallium acetate, ibrilliant green Jcrystal violet.

All treponemes were resistant to cycloserine (500 to 1,000 µg./ml.), polymyxin B sulphate (500 to > 1,000 units/ml.), nitrofurazone (100 to 1,000 μ g./ml.), sulphathiazole (1,000 to >1,000 μ g./ml.), sulphaquinoxaline (1,000 to >1,000 µg./ml.), sulphadiazine (1,000 to >1,000 μ g./ml.), succinyl sulphathiazole (1,000 to >1,000 μg./ml.), nalidixic acid (500 to > 1,000 µg./ml.), methenamine mandelate (500 to >1,000 μ g./ml.), 5-aminouracil (>1,000 μ g./ml.), 5-iodouracil (>1,000 μ g./ml.), azocarmine (500 to > 1,000 μ g./ml.), indigocarmine (1,000 to $> 1,000 \mu g./ml.$), toluidine blue O (500 to > 1,000 μ g./ml.), lysozyme (> 1,000 μ g./ml.), and lysostaphin $(>1,000 \mu g./ml.)$.

Discussion

To interpret the data on the sensitivity of treponemes to antimicrobial agents, certain parameters have been adopted as a guide to be used in determining which antibiotic concentrations are inhibitory (Abramson, 1971). Antimicrobial agents that inhibited the growth of treponemes at 10 µg. or units/ml. of medium or below were considered to be those to which the treponemes were sensitive; however, most sensitive strains were inhibited by 1 unit or µg./ml. or less. Some antibiotic concentrations obtainable in blood serum were listed by Busch and Lane (1967).

All treponemes studied were considered to be sensitive to erythromycin, bacitracin and cephalothin. Most strains were sensitive to the penicillins, vancomycin, tylosin, lincomycin, tetracyclines, and gentamicin. All the serum requiring isolates and the rumen fluid requiring treponemes from pig intestinal tracts were sensitive to the penicillins. Serum requiring treponemes were more sensitive to penicillin than the rumen fluid requiring intestinal strains. Strains requiring rumen fluid which were taken from the human oral cavity were generally resistant to the penicillins.

Only treponemes isolated from pig faeces were resistant to vancomycin and the tetracyclines. All other isolates were sensitive to these antibiotics. Resistance of pig faecal treponemes to tetracyclines could be due to the mixing of so-called growth stimulating levels of tetracycline antibiotics in animal feeds.

All strains studied were resistant to cycloserine, polymyxin B sulphate, nitrofurazone, sulphonamides, nalidixic acid, methenamine mandelate, 5-aminouracil, 5-iodouracil, azocarmine, indigocarmine, toluidine blue O, lysozyme, and lysostaphin. Most strains were resistant to chloramphenicol, streptocmycin, and dihydrostreptomycin.

Growth inhibitory concentrations of antimicrobial agents for various treponemes reported in this investigation were comparable to results of previous investigators. Good agreement was generally found for penicillin (Fitzgerald and Hampp, 1952; Hampp and Fitzgerald, 1959), erythromycin (Keller and Morton, 1953; Berger, 1956; Hampp and Fitgerald, 1959) and tetracyclines (Fitzgerald and Hampp, 1952; Berger, 1956). Our results showed higher inhibitory concentrations of chloramphenicol and tyrothricin (Fitzgerald and Hampp, 1952) and polymyxin (Berger, 1956; Hampp and Fitgerald, 1959). These differences may be accounted for by our use of better anaerobic techniques than were used by previous investigators, thus allowing for rapid and heavy growth of treponemes.

Summary

The sensitivities of seventeen strains of cultivable treponemes representing most of the known species and as yet unidentified isolates from pig faeces and the human oral cavity to fifty antimicrobial agents were determined. The treponemes studied were all sensitive to cephalothin, bacitracin and erythromycin and most strains were sensitive to the penicillins, vancomycin, tylosin, lincomycin, tetracycline, and gentamicin.

All strains studied were resistant to cycloserine, polymyxin B sulphate, nitrofurazone, sulphonamides, nalidixic acid, methenamine mandelate, 5-aminouracil, 5-iodouracil, azocarmine, indigocarmine, tolui-

dine blue O, lysozyme, and lysostaphin. Most strains were resistant to chloramphenicol, streptomycin, dihydrostreptomycin, kanamycin, neomycin, viomycin, tyrothricin, furazolidone, usnic acid, 5-fluorouracil, K tellurite, thallium acetate, brilliant green, and crystal violet. Treponemes varied in their sensitivity to novobiocin.

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Inhibition de la croissance des tréponèmes par les agents antimicrobiens

SOMMAIRE

On a déterminé les sensibilités, vis-à-vis de 50 agents antimicrobiens, de 17 souches de tréponèmes cultivables, représentant la plupart des espèces connues, aussi bien que de souches non identifiées isolées des fèces du porc et de la cavité buccale humaine. Les tréponèmes étudiés furent tous sensibles à céphalothine, bacitracine, et érythromycine, et la plupart des souches furent sensibles à pénicilline, vancomycine, tylosine, lincomycine, tétracycline et gentamicine.

Toutes les souches étudiées furent résistantes à cyclosérine, sulfate de polymyxine B, nitrofurazone, sulfamides, acide nalidixique, mandélate de méthamine, 5-aminouracil, 5-iodo- uracil, azocarmine, indigocarmine, bleu de toluidine O, lyzozyme et lysostaphine. La plupart des souches furent résistantes à chloramphénicol, streptomycine, dihydrostreptomycine, kanamycine, néomycine, viomycine, tyrothricine, furazolidone, acide usnique, 5fluorouracile, tellurite K, acétate de thallium, vert brillant et cristal violet. La sensibilité à la novobiocine était variable selon la souche de tréponème considérée.